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(54) A leader sequence to promote the secretion of gene products.

(57) A recombinant DNA transfer vector contains a leader sequence polynucleotide which codes for a signal polypeptide of formula I,

Met – Arg – Pro – Ser – Ile – His – Arg – Thr –
Ala – Ile – Ala – Ala – Val – Leu – Ala – Thr –   I
Ala – Phe – Val – Ala – Gly – Thr

Preferably the transfer vector is a plasmid. In one preferred embodiment the leader sequence polynucleotide is downstream of and in reading phase with a bacterial or yeast promotor and a ribosome binding site, and upstream of and in reading phase with a structural gene. The structural gene may be, for example, the carboxypeptidase G_2(CPG_2) gene from the chromosomal DNA of Pseudomonas species strain RS – 16. Examples of plasmids containing the leader sequence polynucleotide and the CPG_2 gene are pNM1, pNM111, pNM14, pNM21, pNM22, pNM31, pNM32 and pLEC3.

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LEADER SEQUENCE TO PROMOTE THE SECRETION OF GENE PRODUCTS

The present invention relates to fragments of specific deoxyribonucleotide sequences that promote the secretion of gene products from cells and in particular to recombinant DNA transfer vectors that contain these fragments.

Recent developments in biochemistry have led to the construction of recombinant DNA transfer vectors in which, transfer vectors, for example plasmids, are made to contain exogeneous DNA. In some cases the recombinant incorporates heterologous DNA that codes for polypeptides that are ordinarily not produced by the organism susceptible to transformation by the recombinant vehicle.

In its basic outline a method of endowing a micro organism with the ability to synthesise a new protein involves three general steps:

(a) isolation and purification of the specific gene or nucleotide sequences containing the genetically coded information for the amino acid sequence of the desired protein or polypeptide,

(b) recombination of the isolated gene or nucleotide sequence with an appropriate transfer vector, typically DNA of a bacteriophage or plasmid to form a recombinant transfer vector that codes, in part, for the production of the desired protein or polypeptide,

(c) transfer of the vector to the appropriate micro organism and selection of a strain of the recipient micro organism containing the desired genetic information.

Provided the gene or nucleotide sequence expresses its protein or polypeptide in the chosen micro organism, growth of the micro organism should then produce the desired protein or polypeptide in significant quantities.

Once the micro organism has been cultured, the protein or polypeptide must be isolated from the undesired materials. This step is considerably facilitated if the majority of the desired protein or polypeptide is present in the culture medium and/or the periplasmic space of the micro organism. In other words purification may be performed in a more efficient manner if, once expressed, the protein or polypeptide passes through the cell membrane and out of the cytoplasm.

The passage of the protein or polypeptide through the cell membrane is desirable for two main reasons. First the desired protein or polypeptide will generally be foreign to the micro organism in which it is expressed. In many cases, therefore, it will be quickly broken down by proteolytic enzymes etc in the cells cytoplasm and will, subsequently, have a short half life within the cell. By transferring the protein or polypeptide out of the cytoplasm soon after expression the stability of the protein or polypeptide will be greatly increased. Second the number of unwanted genetic materials and products (from which the desired protein or polypeptide must be isolated) will be far greater in the cell's cytoplasm than in the culture medium and/or in the cell's periplasmic space. It can be seen that on both of the above counts the transfer of the protein or polypeptide through the cell membrane and out of the cytoplasm will greatly facilitate protein or polypeptide isolation.

One way in which the secretion of gene products from the cell's cytoplasm may be promoted is to produce, within the cytoplasm, a preprotein or prepolypeptide in which the desired protein or polypeptide is preceded by a signal polypeptide. The predominantly hydrophobic signal polypeptide directs the desired protein or polypeptide to the cell's periplasmic space, where the signal peptide is removed as the desired protein or polypeptide

traverses the cell membrane.

Many of the known signal peptides contain cysteine residues. These residues have been found to react in the cell membrane and thereby inhibit the efficient transfer of the desired gene product out of the cell.

It is the primary object of the present invention to provide recombinant DNA transfer vectors containing a leader sequence polynucleotide that codes for a signal peptide that is cysteine free. Other objects and advantages of the present invention will become apparent from the following description thereof.

According to the present invention there is provided a recombinant DNA transfer vector comprising a leader sequence polynucleotide coding for signal polypeptide of formula I,

Met-Arg-Pro-Ser-Ile-His-Arg-Thr-Ala-Ile-Ala-
Ala-Val-Leu-Ala-Thr-Ala-Phe-Val-Ala-Gly-Thr   I

The transfer vector may be a bacteriophage or, which is preferred, a plasmid.

Preferably the majority of the codons in the nucleotide sequence are those preferred for the expression of microbial genomes. Suitable codons are listed in UK 1,568,047 and UK 2007675A, and these publications are therefore incorporated herein by reference.

In one preferred embodiment of the present transfer vector the nucleotide sequence has formula II

5'- ATG CGC CCA TCC ATC CAC CGC ACA
GCC ATC GCC GCC GTG CTG GCC ACC   II
GCC TTC GTG GCG GGC ACC - 3'

The nucleotide sequence coding for the signal polypeptide (the leader sequence poly nucleotide) will preferably be downstream of and in reading phase with a bacterial or a yeast promoter and a prokaryotic ribosome binding site in the transfer vector. Moreover the leader sequence polynucleotide will either be upstream of an insertion site for a structural gene or, which is preferred, will be upstream of and in reading phase with a structural gene coding for a desired protein or polypeptide. Preferably the gene codes for a eukaryotic, particularly a mammalian, protein or polypeptide.

The structural gene may code, for example, for such eukaryotic proteins as human growth hormone, human insulin or human chorionic somatomammotropin. Alternatively it may code for such prokaryotic

proteins as E.coli β-galactosidase or Pseudomonas carboxy peptidase G_2 (CPG_2) (Carboxypeptidase G_2 is an enzyme, produced by Pseudomonas species strain RS-16, that has application in cancer chemotherapy. It is a Zn^{2+} containing dimer of 2 x 42,000 daltons and has high affinities (Km values of 10^{-5} or 10^{-6}M) for both 5-methyltetrahydrofolate, the predominant ciculatory form of folate in mammals and for the folic acid antagonist methotrexate (MTX), which is widely used in cancer chemotherapy. The enzyme may be used directly for the plasma depletion of reduced folates, essential as co-factors in purine and particularly in pyrimidine biosynthesis. CPG_2 has been shown to inhibit the development of the Walker 256 carcinoma in vivo and to remove MTX from circulation in patients where prolonged exposure to high doses of MTX leads to toxicity).

Examples of transfer vectors according to the present invention that code for CPG_2 are pNM1, pNM111, pNM14, pNM21, pNM22, pNM31, pNM32 and pLEC3.

The promoter is preferably a high expression bacterial or yeast promoter for the structural gene in a variety of hosts. The particular choice of promoter will depend on the microorganism to be transformed. For example the transformation of E.coli will generally be effected by a transfer vector in which an E.coli promoter controls the expression of the structural gene. Examples of E.coli promoters are those present in the plasmids pBR 322 and pAT 153. By contrast, the transformation of Pseudomonas species will generally be effected by a transfer vector in which a Pseudomonas promoter controls the expression of the structural gene. Examples of Pseudomonas promoters are those present in the plasmid pKT 230 or Pseudomonas chromosomal DNA.

In order to express the structural gene the present transfer vector will be transformed into a suitable microorganism. According to a further aspect of the present invention therefore there is provided a microorganism transformed by a recombinant DNA transfer vector according to this invention. The microorganism will preferably be a bacterium or yeast in which high expression of the structural gene, within the transfer vector, occurs. Depending on the choice of promoter the microorganism may be a strain chosen from one of the following bacteria E.coli, Pseudomonas and Bacillus or the yeast Saccharomyces cerevisiae.

Having transformed the microorganism, the protein or polypeptide, for which the structural gene codes, may then be expressed by culturing

the transformed microorganism in a culture medium. It is the primary advantage of the present invention that culturing the transformed microorganism affords a preprotein or prepolypeptide in which the desired protein or polypeptide is preceded by the present signal polypeptide. This means that soon after expression the signal polypeptide directs the desired protein or polypeptide to the cell's periplasmic space, where the signal polypeptide is removed as the desired protein or polypeptide traverses the cell membrance. Since the present signal polypeptide is free of cysteine residues the desired gene product will be efficiently secreted through the membrane.

The present transfer vectors may be prepared by any of the methods that are well known in the recombinant DNA art. For example the leader sequence poly nucleotide may be synthesised by the modified triester method of K.Itakura etal, JACS, 1975, 97, 7327 or by the improved oligodeoxynucleotide preparation described in UK 2007675A. The disclosure of both of these references is incorporated herein by reference. The synthesised polynucleotide may then be inserted in a transfer vector, preferably a plasmid. In the transfer vector it will preferably be downstream of and in reading phase with a bacterial or a yeast promoter and a prokaryotic ribosome binding site. The leader sequence polynucleotide should also be either upstream of a structural gene insertion site or upstream of and in reading phase with a structural gene.

Alternatively, DNA fragments containing the leader sequence polynucleotide may be obtained from natural sources, in particular from the chromosomal DNA of Pseudomonas species strain RS-16. In this particular case a polynucleotide (formula II above) coding for the present signal polypeptide immediately precedes a structural gene coding for CPG_2. A number of the DNA fragments containing this leader sequence polynucleotide may therefore be recognised by their ability, on insertion into a plasmid and transformation of a microorganism by the resultant recombinant vector, to enable a microorganism to grow on folate. Examples of such recombinant transfer vectors that contain both a polynucleotide coding for the present signal polypeptide(formula II above) and a structual gene coding for CPG_2 are pNM1, pNM111, pNM14, pNM21, pNM22, pNM31, pNM32 and pLEC3. Of course, once a Fol^+ recombinant vector has been obtained in this way it may be subcloned to afford alternative vectors (either Fol^+ or Fol^-) that also contain a polynucleotide coding for the present signal polypeptide.

Once a suitable DNA fragment has been isolated it may then be inserted in a transfer vector, preferably a plasmid. In the transfer vector the leader sequence polynucleotide on the inserted fragment should be downstream of and in reading phase with a bacterial or a yeast promoter and a prokaryotic ribosome binding site. The leader sequence polynucleotide should also be either upstream of a structural gene insertion site or upstream of and in reading phase with a structural gene.

The structural gene for insertion downstream of and in reading phase with the present leader sequence polynucleotide may be obtained, for example, by the synthetic methods mentioned above (this is particularly useful for the preparation of genes coding for small proteins, such as human growth hormone, insulin and human chorionic somatomammotropin.) Alternatively the structural gene may be prepared from m-RNA by the use of the enzyme reverse transcriptase or may be isolated from natural sources (chromosomal DNA).

An example of the latter method is the isolation of DNA fragments containing a polynucleotide sequence (shown in Table 1) coding for the enzyme CPG_2 (amino acid sequence also shown in Table 1) from Pseudomonas species strain RS-16 chromosomal DNA. Examples of plasmids containing a CPG_2 structural gene, as well as a polynucleotide coding for the present signal polypeptide (formula II above), are pNM1, pNM111, pNM14, pNM21, pNM22, pNM31, pNM32 and pLEC3.

Once prepared or isolated the Leader sequence polynucleotide and the structural gene will be inserted into a transfer vector, preferably a plasmid, to form a recombinant DNA transfer vector according to the present invention. The insertion step or steps will preferably be effected by one of the well known techniques in this art that employ restriction endonucleases, see for example the methods discussed in UK 2090600A, the disclosure of which is incorporated herein by reference. The choice of transfer vector will be determined by the microorganism in which the leader sequence polynucleotide and structural gene are to be expressed. Generally the transfer vector will be a cloning vehicle that is suitable for transforming the chosen micro-organisms and that displays a phenotypical characteristic, such as antibiotic resistance, by which the recombinant transfer vectors may be selected. Thus, if the micro-organism is to be E-coli, then suitable transfer vectors will be the E-coli plasmids pBR322 and pAT153. Alternatively, if the micro-organism is to be Pseudomonas,

TABLE 1

A Polynucleotide Sequence, coding for CPG_2, isolated from Pseudomonas species strain RS - 16 chromosomal DNA

	1 Met	Arg	Pro	Ser	Ile	His	Arg	Thr
5' -	ATG	CGC	CCA	TCC	ATC	CAC	CGC	ACA
Ala	10 Ile	Ala	Ala	Val	Leu	Ala	Thr	Ala
GCC	ATC	GCC	GCC	GTG	CTG	GCC	ACC	GCC
Phe	Val	20 Ala	Gly	Thr	Ala	Leu	Ala	Gln
TTC	GTG	GCG	GGC	ACC	GCC	CTG	GCC	CAG
Lys	Arg	Asp	30 Asn	Val	Leu	Phe	Gln	Ala
AAG	CGC	GAC	AAC	GTG	CTG	TTC	CAG	GCA
Ala	Thr	Asp	Glu	40 Gln	Pro	Ala	Val	Ile
GCT	ACC	GAC	GAG	CAG	CCG	GCC	GTG	ATC
Lys	Thr	Leu	Glu	Lys	50 Leu	Val	Asn	Ile
AAG	ACG	CTG	GAG	AAG	CTG	GTC	AAC	ATC
Glu	Thr	Gly	Thr	Gly	Asp	60 Ala	Glu	Gly
GAG	ACC	GGC	ACC	GGT	GAC	GCC	GAG	GGC
Ile	Ala	Ala	Ala	Gly	Asn	Phe	70 Leu	Glu
ATC	GCC	GCT	GCG	GGC	AAC	TTC	CTC	GAG
Ala	Glu	Leu	Lys	Asn	Leu	Gly	Phe	80 Thr
GCC	GAG	CTC	AAG	AAC	CTC	GGC	TTC	ACG
Val	Thr	Arg	Ser	Lys	Ser	Ala	Gly	Leu
GTC	ACG	CGA	AGC	AAG	TCG	GCC	GGC	CTG
90 Val	Val	Gly	Asp	Asn	Ile	Val	Gly	Lys
GTG	GTG	GGC	GAC	AAC	ATC	GTG	GGC	AAG

	100							
Ile	Lys	Gly	Arg	Gly	Gly	Lys	Asn	Leu
ATC	AAG	GGC	CGC	GGC	GGC	AAG	AAC	CTG
		110						
Leu	Leu	Met	Ser	His	Met	Asp	Thr	Val
CTG	CTG	ATG	TCG	CAC	ATG	GAC	ACC	GTC
			120					
Tyr	Leu	Lys	Gly	Ile	Leu	Ala	Lys	Ala
TAC	CTC	AAG	GGC	ATT	CTC	GCG	AAG	GCC
				130				
Pro	Phe	Arg	Val	Glu	Gly	Asp	Lys	Ala
CCG	TTC	CGC	GTC	GAA	GGC	GAC	AAG	GCC
					140			
Tyr	Gly	Pro	Gly	Ile	Ala	Asp	Asp	Lys
TAC	GGC	CCG	GGC	ATC	GCC	GAC	GAC	AAG
						150		
Gly	Gly	Asn	Ala	Val	Ile	Leu	His	Thr
GGC	GGC	AAC	GCG	GTC	ATC	CTG	CAC	ACG
							160	
Leu	Lys	Leu	Leu	Lys	Glu	Tyr	Gly	Val
CTC	AAG	CTG	CTG	AAG	GAA	TAC	GGC	GTG
								170
Arg	Asp	Tyr	Gly	Thr	Ile	Thr	Val	Leu
CGC	GAC	TAC	GGC	ACC	ATC	ACC	GTG	CTG
Phe	Asn	Thr	Asp	Glu	Glu	Lys	Gly	Ser
TTC	AAC	ACC	GAC	GAG	GAA	AAG	GGT	TCC
180								
Phe	Gly	Ser	Arg	Asp	Leu	Ile	Gln	Glu
TTC	GGC	TCG	CGC	GAC	CTG	ATC	CAG	GAA
	190							
Glu	Ala	Lys	Leu	Ala	Asp	Tyr	Val	Leu
GAA	GCC	AAG	CTG	GCC	GAC	TAC	GTG	CTC
		200						
Ser	Phe	Glu	Pro	Thr	Ser	Ala	Gly	Asp
TCC	TTC	GAG	CCC	ACC	AGC	GCA	GGC	GAC

			210					
Glu	Lys	Leu	Ser	Leu	Gly	Thr	Ser	Gly
GAA	AAA	CTC	TCG	CTG	GGC	ACC	TCG	GGC
				220				
Ile	Ala	Tyr	Val	Gln	Val	Asn	Ile	Thr
ATC	GCC	TAC	GTG	CAG	GTC	AAC	ATC	ACC
					230			
Gly	Lys	Ala	Ser	His	Ala	Gly	Ala	Ala
GGC	AAG	GCC	TCG	CAT	GCC	GGC	GCC	GCG
						240		
Pro	Glu	Leu	Gly	Val	Asn	Ala	Leu	Val
CCC	GAG	CTG	GGC	GTG	AAC	GCG	CTG	GTC
							250	
Glu	Ala	Ser	Asp	Leu	Val	Leu	Arg	Thr
GAG	GCT	TCC	GAC	CTC	GTG	CTG	CGC	ACG
								260
Met	Asn	Ile	Asp	Asp	Lys	Ala	Lys	Asn
ATG	AAC	ATC	GAC	GAC	AAG	GCG	AAG	AAC
Leu	Arg	Phe	Asn	Trp	Thr	Ile	Ala	Lys
CTG	CGC	TTC	AAC	TGG	ACC	ATC	GCC	AAG
270								
Ala	Gly	Asn	Val	Ser	Asn	Ile	Ile	Pro
GCC	GGC	AAC	GTC	TCG	AAC	ATC	ATC	CCC
	280							
Ala	Ser	Ala	Thr	Leu	Asn	Ala	Asp	Val
GCC	AGC	GCC	ACG	CTG	AAC	GCC	GAC	GTG
		290						
Arg	Tyr	Ala	Arg	Asn	Glu	Asp	Phe	Asp
CGC	TAC	GCG	CGC	AAC	GAG	GAC	TTC	GAC
			300					
Ala	Ala	Met	Lys	Thr	Leu	Glu	Glu	Arg
GCC	GCC	ATG	AAG	ACG	CTG	GAA	GAG	CGC
				310				
Ala	Gln	Gln	Lys	Lys	Leu	Pro	Glu	Ala
GCG	CAG	CAG	AAG	AAG	CTG	CCC	GAG	GCC

					320			
Asp	Val	Lys	Val	Ile	Val	Thr	Arg	Gly
GAC	GTG	AAG	GTG	ATC	GTC	ACG	CGC	GGC
						330		
Arg	Pro	Ala	Phe	Asn	Ala	Gly	Glu	Gly
CGC	CCG	GCC	TTC	AAT	GCC	GGC	GAA	GGC
							340	
Gly	Lys	Lys	Leu	Val	Asp	Lys	Ala	Val
GGC	AAG	AAG	CTG	GTC	GAC	AAG	GCG	GTG
								350
Ala	Tyr	Tyr	Lys	Glu	Ala	Gly	Gly	Thr
GCC	TAC	TAC	AAG	GAA	GCC	GGC	GGC	ACG
Leu	Gly	Val	Glu	Glu	Arg	Thr	Gly	Gly
CTG	GGC	GTG	GAA	GAG	CGC	ACC	GGC	GGC
360								
Gly	Thr	Asp	Ala	Ala	Tyr	Ala	Ala	Leu
GGC	ACC	GAC	GCG	GCC	TAC	GCC	GCG	CTC
	370							
Ser	Gly	Lys	Pro	Val	Ile	Glu	Ser	Leu
TCA	GGC	AAG	CCA	GTG	ATC	GAG	AGC	CTG
		380						
Gly	Leu	Pro	Gly	Phe	Gly	Tyr	His	Ser
GGC	CTG	CCG	GGC	TTC	GGC	TAC	CAC	AGC
			390					
Asp	Lys	Ala	Glu	Tyr	Val	Asp	Ile	Ser
GAC	AAG	GCC	GAG	TAC	GTG	GAC	ATC	AGC
				400				
Ala	Ile	Pro	Arg	Arg	Leu	Tyr	Met	Ala
GCG	ATT	CCG	CGC	CGC	CTG	TAC	ATG	GCT
					410			
Ala	Arg	Leu	Ile	Met	Asp	Leu	Gly	Ala
CGC	CGC	CTG	ATC	ATG	GAT	CTG	GGC	GCC
Gly	Lys							
GGC	AAG	TGA - 3'						

Amino acids 1 to 22 are the present signal polypeptide
Amino acids 23 to 415 are the CPG_2 structural gene

NB The leader sequence polynucleotide is the preferred polynucleotide of formula II.

then a suitable transfer vector will be Pseudomonas pkT230.

The present recombinant DNA transfer vectors, micro-organisms transformed by the present recombinant DNA transfer vectors and processes for the preparation of said vectors and micro-organisms will now be described by way of example only, with particular reference to the Figures in which:

Figure 1 is a restriction enzyme cleavage site map of pNM1,
Figure 2 is a restriction enzyme cleavage site map of pNM111,
Figure 3 is a restriction enzyme cleavage site map of pNM14,
Figure 4 is a restriction enzyme cleavage site map of pNM21,
Figure 5 is a restriction enzyme cleavage site map of pNM22, and
Figure 6 illustrates the process for the preparation of a recombinant plasmid containing both the present leader sequence polynucleotide and the β-Galactosidase structural gene, and
Figure 7 is a restriction enzyme cleavage site map of pLEC3.

Materials and Methods

Bacterial strains and plasmids

The bacterial strains used were Escherichia coli W5445 (pro leu thi thr^{o} sup E44 lac Y ton A r^{-} m^{-} Str^{r}) Pseudomonas putida 2440 (r^{-}) and Pseudomonas sp strain RS-16. The plasmids employed were pBR322 (F Bolivar et al Gene, 1977, 2, 95), pAT153 (A J Twigg et al, Nature, 1980, 283, 216) and pKT230 (M Bagdasarin et al, Gene 1981, 16, 237) and pROG5 (R.F.Sherwood et al, The Molecular Biology of Yeast,1979 Cold Spring Harbor Publications).

Media and culture conditions

E.coli was routinely cultured in L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Solidified medium (L-agar) consisted of L-broth with the addition of 2% (w/v) agar (Bacto-Difco). Antibiotic concentrations used for the selection of transformants were 50 μg/ml ampicillin, 15 μg/ml tetracycline and 30 μg/ml kanamycin. In the case of E.coli these were conducted in 2YT liquid medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) containing 1% glucose, and 0.05% folate where appropriate. The pseudomonads were grown in a minimal salts solution consisting of per litre: $MgSO_4$, 0.05g; $CaCl_2$, $2H_2O$, 0.05g; $FeSO_4.7H_2O$, 0.005g; $MnSO_4$, 0.0015g; Na_2Mbo_4, $2H_2O$, 0.0015g; KH_2PO_4; 5g; $K_2HPO_4.3H_2O$, 12g; glutamate, 10g. The minimal medium employed for E.coli was M9 medium (J Miller, Experiments in molecular genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1972).

Purification of DNA

Plasmids were purified from chloramphenicol amplified cultures (D B Clewell, J Bacteriol, 1972, 110, 667) by Brij-lysis (D B Clewell et al, Proc Natl Acad Sci, USA, 1969, 62, 1159) and subsequent caesium

chloride-ethidium bromide density gradient centrifugation (A Colman et al, Eur.J Biochem, 1978, 91, 303). A rapid, small scale plasmid isolation technique (Burnboim et al, Nuc.Acids Res, 1979, 7, 1513) was also employed for screening purposes. Chromosomal DNA from the donor Pseudomonad strain (RS-16) was prepared essentially as described by J Marmar, J.Mol.Biol, 1961, 3, 208.

Restriction, ligation and transformation methods

Restriction endonucleases and DNA ligase were purchased from Bethesda Research Laboratories and used in the buffers and under the conditions recommended by the supplier. Transformation of E.coli was essentially as described by S N Cohen et al., Proc.Natl.Acad.Sci., USA, 1972, 69, 2110, while Ps.putida was transformed by the method of M Bagdasarian and K N Timmis, Current Topics in Microbiology and Immunology, Eds P H Hofschneider and W Goebel, Springer Verlag, Berlin, 1981, p 47.

Agarose gel electrophoresis

Digests were electrophoresed in 0.8% agarose slab gels (10 cm x 20 cm x 0.5 cm) on a standard vertical system (Raven), employing Tris-borate-EDTA buffer. Electrophoresis of undigested DNA was at 125V, 50 mA for 3 hours, while digested DNA was electrophoresed at 15V, 10 mA for 16 hours. Fragment sizes were estimated by comparison with fragments of λDNA digested with HindIII and λDNA cut with both HindIII and EcoRI. Fragments were isolated from gels using electroelution (M W McDonnell et al, Proc. Natl.Acad.Sci, USA, 1977, 74, 4835).

Determination of carboxypeptidase G_2 activity

Bacteria were grown in 1 litre batch culture and 100 ml samples taken at various stages in the growth phase. Samples were cooled on ice, centrifuged at 13,000 x g for 10 minutes and resuspended and frozen in 5 ml of 0.1 M Tris HCl, pH 7.3 containing 0.2 mM $ZnSO_4$. The cells were disrupted using a MSE Ultrasonic Disintegrator (150 W) at medium frequency, amplitude 2, for three 30-second intervals on ice. Cell debris was removed by centrifugation at 10,000 x g for 5 minutes. CPG_2 activity was determined after J L McCullough et al, J.Biol.Chem, 1971, 246, 7207. A 1ml reaction cuvette containing 0.9 ml of 0.1 M Tris-HCl, pH 7.3 plus 0.2 mM $ZnSO_4$ and 0.1 ml of 0.6 mM methotrexate was equilibrated at 37°C. Enzyme extract was added to the test cuvette and the decrease in absorbance at 320 nm measured using a Pye-Unicam SP1800 double-beam spectrophotometer. Enzyme activity per ml extract

was calculated as Δ 320 nm absorbance/min divided by 8.3, which is equivalent to the hydrolysis of 1 μmol of MTX/min at 37°C. Protein concentration was determined by the method of M M Bradford, Anal Biochem, 1976, 72, 248.

Cell fractionation techniques

Bacterial cultures were grown in the low phosphate medium of H C Neu and L A Heppel, (J Biol Chem, 1964, 240, 3685), supplemented with 100 μg/ml ampicillin, to an OD_{450} = 1.0. 40 ml of culture was centrifuged at 5000 g for 10 min, washed in 5 ml of 10 mM Tris-HCl pH 7.0, and resuspended in 0.9 ml 0.58 M sucrose, 0.2 mM DTT, 30 mM Tris-HCl pH 8.0. Conversion to spheroplasts was achieved by the addition of 20 μl of lysozyme (2 mg/ml), 40 μl 0.1 M EDTA, and incubation at 23° for 10 min (HC Neu et al, J Biol Chem, 1964, 239, 3893). The spheroplasts were placed on ice and 0.1 ml of 30% (w/v) BSA added, followed by 5 ml of sucrose-tris buffer. Sedimentation of the spheroplasts was achieved by centrifugation at 5000g for 10 min and the supernatant retained as the 'periplasmic' fraction. The pellet was resuspended in 5 ml 10 mM Tris-HCl, 0.2 mM DTT pH 7.0 and and sonicated at 20 Kc/sec, 2 Amps for 15 sec. Remaining whole cells were removed by centrifugation at 1000 x g for 10 min. Centrifugation at 100000 x g for 1 hr, at 4°C, separated the soluble (cytoplasmic) proteins from the particulate (membrane-bound) proteins. The membrane pellet was resuspended in 1 ml of 10mM Tris-HCl, 0.2 mM DTT, pH 7.0.

CPG_2 was assayed as described. Alkaline phosphatase was assayed according to J Miller, Experiments in Molecular genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1972, NADH oxidase according to M J Osborn et al, J Biol Chem, 1972, 247, 3962 and glyceraldehyde - 3 - phosphate dehydrogenase after K Suzuki et al, FEMS, 1971, 13, 217.

Example 1

Preparation of recombinant plasmid pNM1 (A plasmid containing both the present leader sequence polynucleotide and the CPG_2 structural gene)

To isolate the gene for carboxypeptidase G_2 together with the leader sequence polynucleotide chromosomal DNA prepared from the Pseudomonas host (strain RS-16) was partially digested with Sau3A and fragments of between 6-8 Md isolated from agarose gels by electroelution. The 'sized' DNA was ligated with alkaline phosphatase treated BamH1 cut pBR322, transformed into E.coli W5445, and Ap^r transformants selected. Of the 3,500 Ap^r colonies obtained, approximately 70% were TC^s. Utilisation of a rapid plasmid isolation technique on 50 Ap^r TC^s transformants demonstrated that 90% of the gene bank harboured plasmids of the expected size. As a further check on the authenticity of the gene bank, the individual clones were screened for the acquisition of a Leu^+ phenotype. Two such clones were identified. Both carried a plasmid capable of transforming leuB (B-isopropylmalate dehydrogenase) E.coli mutants to prototrophy.

Acquisition of a functional CPG_2 gene should enable E.coli to utilise folic acid as a carbon source. The 2,400 gene bank clones were screened for the ability to grow on minimal medium containing folate as the sole source of carbon (ie Fol^+). A single Fol^+ clone was detected and shown to harbour a plasmid capable of transforming plasmid-minus W5445 to the Fol^+ phenotype. Classical restriction mapping of this plasmid (pNM1) was undertaken which revealed the presence of a 5.9 Md insert of pseudomonad DNA within pBR322. The restriction enzyme clearage site map of pNM1 is given in Figure 1. The nucleotide sequence of the leader sequence polynucleotide and the CPG_2 structural gene is given in Table 1.

Example 2

Subcloning of plasmid pNM1 to form pNM111

In order to pinpoint the position of the CPG_2 gene and the leader sequence polynucleotide within the 5.9 Md insert, subcloning of various restriction enzyme fragments, into pBR322, was undertaken. A functional CPG_2 gene was shown not to occur on

Xhol or Sphl fragments of tne pNMl insert, but was present on a 3.1 Md Bglll fragment. This latter fragment was cloned into the BamHl site of pBR322 to give pNMl1 (6.0 Md). A further reduction in the size of pNMl1 was achieved by digesting with Sall and religating the resultant fragment to yield pNMl11. In addition, plasmids in which the smaller 0.95 Md Sall fragment had become inserted in the opposite orientation to the parent plasmid (pNMl1) were Fol^-. Taken together these subcloning results indicate that the CPG_2 gene and the leader sequence polynucleotide lie between the Bglll site at 4.14 and the SAll site at 6.03 on pNMl. Furthermore, the gene contains a Sphl (5.17), Sall (5.07) and at least one Xhol (4.56 and/or 5.56) site. The restriction enzyme cleavage site map of pNMl11 is given in Figure 2.

Example 3

Preparation of recombinant plasmid pNM14. (A plasmid containing both the present leader sequence polynucleotide and the CPG_2 structural gene)

The 3.1 Md Bgl II fragment from Example 2 above was partially digested with Sau3A. These fragments were then cloned into the Bam HI site of pAT153 and transformed into E coli W5445. Of the two Ap^r Tc^s Fol^+ colonies obtained, one contained a plasmid which had acquired an extra Sal I and Bam HI site, this was pNM 14. The restriction enzyme cleavage site map of pNM 14 is given in Figure 3. Sequencing of the leader sequence polynucleotide and the CPG_2 structural gene present in pNM 14 gave the nucleotide structure shown in Table 1. DNA sequencing of pNM 14 also revealed that the Sal I - Bam HI fragment was a duplication of a segment of DNA from within the insert (marked * on Figure 3) composed of two contiguous Sau 3A fragments.

Example 4 and 5

Preparation of recombinant plasmids pNM 21 and pNM 22 (Plasmids containing both the present leader sequence polynucleotide and the CPG_2 structural gene)

The 3.1 Md Bgl II fragment from Example 2 was cloned into the Bam HI site of pAT 153 and transformed into E coli W 5445. Two Ap^r Tc^s Fol^+ colonies were obtained, one containing a plasmid pNM 21 in which the fragment was inserted in the opposite orientation to

pNM1 and one containing a plasmid pNM22 in which the fragment was inserted in the same orientation as pNM1. The restriction enzyme cleavage site maps of pNM21 and pNM22 are given in Figures 4 and 5 respectively.

The two plasmids, pNM21 and pNM22 both transformed E.coli to Fol^+, indicating that a pseudomonad promoter was present on the 3.1Md fragment. However, cells carrying the plasmid pNM21, in which the Bglll fragment was cloned in the opposite orientation to pNM1, exhibited more rapid growth with folic acid as the sole carbon source. This difference was clearly visible on agar medium, where colonies developed concentric yellow 'halos' of precipitated pteroic acid, the insoluble product of folate hydrolysis.

Confirmation that pNM21 gave enhanced expression of CPG_2 over pNM22, was obtained by assaying enzyme production during batch growth of cells containing either plasmid. (The cells were grown in complex medium supplemented with 1% (w/v) glucose and where appropriate 0.05% (w/v) folic acid. The generation time was 56-66 min. The culture was sampled at hourly intervals and whole cells were disrupted by sonication. Enzyme activity was determined in the centrifugal extract). Results are given in Table 2.

The expression of CPG_2 from the plasmids pNM22 and pNM1 was 2.5 units/litre of culture, representing 0.005% soluble protein. In contrast, expression from pNM21 was 3000-3500 units/litre of culture, which represented 4.7% soluble protein. As the cloned gene is inserted into the BamHI site of pAT153, the observed higher expression of pNM21 is almost certainly due to transcriptional read through from the Tc promoter. The low expression of CPG_2 carried on plasmids pNM1 and pNM22 is consistent with the view that Pseudomonas promoters function poorly in E.coli. It is also apparent from Table 2 that in the presence of folate there is a two-fold increase in the specific activity of enzyme measured in cell sonicates. This phenomenon has been observed in all experiments, but does not seem to be associated with classical induction of the CPG_2 gene, as overall enzyme yield in the presence or absence of folate remains at about 3000 u/litre culture. It in fact reflects a consistent depression in the

soluble protein levels measured in sonicates from cells grown in the presence of folate. There is no obvious difference in growth rate of cells grown with folate and the reasons for this result are not clear.

TABLE 2: CARBOXYPEPTIDASE G PRODUCTION BY E COLI W5445, CONTAINING THE PLASMIDS pNM1, pNM21 and pNM22.

CULTURE AGE (HR)	CARBOXYPEPTIDASE G_2 SPECIFIC ACTIVITY (U/MG SOLUBLE PROTEIN)					
	pNM1		pNM22		pNM21	
	-FOL	+FOL	-FOL	+FOL	-FOL	+FOL
1	-	-	-	-	11.5	13.4
2	-	-	-	-	12.9	9.6
3	.008	.005	.010	.019	13.9	23.3
4	.009	.011	.015	.013	12.3	26.9
5	.007	.019	.016	.016	11.5	25.6
6	.005	.024	.014	.023	13.7	24.1
7	.015	.029	.024	.043	13.2	20.6
8	.013	.028	.024	.046	13.0	23.6

Expression of the cloned gene in *Ps.putida*

The observation that the CPG_2 gene was expressed in *E.coli* regardless of the orientation of the gene within the vector suggested that the promoter region of the CPG_2 gene had been cloned with the structural gene and the leader sequence polynucleotide. The low expression of CPG_2 within *E.coli* from its natural promoter (pNM1, pNM22, pNM111) confirmed other findings that *Pseudomonas* promoters are poorly recognised by *E.coli* RNA polymerases. It would be expected that if the gene was introduced back into a pseudomonad cellular environment, then improved expression from the *Pseudomonas* promoter should result. The 3.1 Md *BglII* fragment was subcloned into the *Pseudomonas* cloning vector pKT230 at its single *BamHI* site.

Two plasmids were obtained, pNM31 and pNM32 representing the two possible orientations of the cloned gene. These plasmids were transformed into Ps.putida 2440 by the method of Bagdasarian and Timmis. Pseudomonad cells carrying both plasmids were cultured in minimal salts medium and enzyme production monitored.

Yields of 500-1000 units/litre of culture were obtained regardless of gene orientation within the plasmid. Specific activity of the enzyme in cell sonicates was 1.5 to 4.0 U/mg protein representing 0.3 to 0.7% soluble protein (compared with $<$ 0.05% soluble protein in the donor strain RS-16). This result strongly indicates that the CPG_2 promoter is present and operating in a pseudomonad background. When the same plasmids were transformed into E.coli W5445 12-40 Units/litre were found at specific activity $<$0.07 U/mg ($<$ 0.01% soluble protein).

Periplasmic localisation of CPG_2

There is evidence that CPG_2 is located in or near the periplasmic space of Pseudomonas strain RS-16. Pteroic acid, the product of CPG_2 hydrolysis of folic acid is extremely insoluble and is found predominantly outside the cell in both liquid and solid media. Exogeneous pteroic acid is also seen in E.coli cultures containing the cloned gene when folic acid is present in the medium. This is clearly demonstrated by the 'halo' of precipitated pteroic acid observed around colonies carrying plasmids in which expression of CPG_2 is from the Tc promoter of pBR322 (eg pNM21).

The localisation of CPG_2 produced by E.coli cells carrying pNM21 was examined by the separation of cellular proteins into cytoplasmic, periplasmic, and whole membrane fractions. As a control, levels of three marker enzymes, alkaline phosphatase (periplasmic), glyceraldehyde-3-phosphate dehydrogenase (cytoplasmic) and NADH.O_2 oxidoreductase (membrane-bound), were also determined. As can be seen from Table 3 97% of the CPG_2 activity occurs in the periplasm, equivalent to the marker periplasmic enzyme, alkaline phosphatase. This confirms the presence in pNM21 of a leader sequence polynucleotide next to the CPG_2 gene that codes for a signal polypeptide according to this in vention that promotes the secretion of CPG_2 from the cytoplasm into the periplasmic space.

Carboxypeptidase G_2 synthesised in E.coli

The specific activity of CPG_2 in crude cell extracts of cells carrying pNM21 was 50-fold higher than equivalent extracts from Pseudomonas strain RS-16. To determine whether the cloned gene-product in E.coli had the same properties as CPG_2 from the pseudomonad, enzyme was purified from E.coli carrying pNM21. The specific activity of purified CPG_2 (single band SDS-PAGE) was 535 U/mg of protein, which compares to 550 U/mg of protein from the pseudomonad. CPG_2 purified from E.coli clone pNM21 co-chromatographed with CPG_2 from Pseudomonas strain RS-16 at a sub-unit molecular weight value of 42,000 daltons. Km vallues using methotrexate as substrate were $7.4 \times 10^{-6}M$ and $8.0 \times 10^{-6}M$ respectively. In addition, antiserum raised against the Pseudomonas enzyme indicated immunological identity between the E.coli and Pseudomonas CPG_2, as a confluent precipitation line was formed on Ouchterlony double diffusion analysis.

TABLE 3

Localisation of Carboxypeptidase

FRACTION	ENZYME ACTIVITY CPG_2	AP	GAPDH	NADHOX
Periplasmic	97.0	97.1	6.8	0.25
Cytoplasmic	2.6	2.3	93	8.4
Membrane-bound	0.4	0.6	0.2	89.1

AP = Alkaline phosphatase

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase

NADHOX = $NADH.O_2$ oxidoreductase

Example 6

Preparation of a recombinant plasmid containing both the present leader sequence polynucleotide and the B-Galactosidase structural gene

Plasmid pNM14 (Example 3) was treated with Sau 3A (GATC) and the fragments were cloned into the Bam HI site of M13 mp7 template DNA (single stranded DNA (Step A of Figure 6). The product carrying a 318bp Sau 3A fragment coding for the present signal polypeptide and the first 22 amino acids of CPG_2 (nucleotide sequence of this fragment shown in Table 4) was selected and made double stranded. The DNA coding for the signal polypeptide (and the first part of CPG_2) was then excised as an Eco RI fragment. This Eco RI fragment was then cloned into the promoter cloning vector E.coli pMC1403 (M.J. Casadaban et al, J Bacteriol, 1980, 143, 971), which carries only the structural gene (lac Z) for B-galactosidase (ie no promoter and no ATG start codon) (Steps B and C of Figure 6). Plasmids were obtained in which the Eco RI fragment had inserted in both orientations, however, only those in which fusion of the CPG_2 sequence to the B-galactosidase sequence had occurred (i) yielded a 0.34 Kb fragment upon digestion with BamHI; (ii) enabled the host cell to hydrolyse the colourless lactose analogue, BCIG, and impart a blue colouration to colonies. The 0.34 Kb BamHI fragment has been recloned into M13mp7 and sequenced to confirm that fusion has occurred. The 'precursor' fusion produced will consist of the signal peptide, the first 22 amino acids of CPG_2, 6 amino acids derived from the M13mp7 and pMC1403linker units, and B-galactosidase from its 8th amino acid onward.

Localisation experiments have been performed on cells carrying a plasmid coding for the 'fusion gene' where the cellular proteins have been fractionated into periplasmic, cytoplasmic and membrane fractions. In these experiments an organism (E,coli MC 1061) which is deleted for the lac Z gene was grown in phosphate medium (H.C. Neu et al, J Biol Chem, 1964, 240, 3685) and periplasmic enzymes were released from the harvested cells by conversion to spheroplasts. Separation of soluble proteins (cytoplasmic) from particulate proteins (membrane band) was achieved by sonicating the harvested

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spheroplasts and subsequent centrifugation at 100,000g for 1hr, to sediment the cell membrane (T.J.Silhary et al, Proc Natl Acad Sci USA, 1976, 73, 3423).

The results given in Table 5 demonstrate the presence of 50% of the B-galactosidase activity in the periplasmic space. This result is in direct contrast to similar work involving fusion of other periplasmic protein signal sequences to B-galactosidase, where the fusion proteins are not exported, but become jammed in the membrane (P.J. Bassfordet al, J Bacteriol, 1979, 139, 19 and S D Emr et al, J Cell, Biol, 1980, 86, 701).

TABLE 4

The Polynucleotide Sequence of the 318 bp Sau 3A Fragment from Recombinant Plasmid pNM14

5' - G	ATC	CAC	GCA	CTG	AAG	GCG	CGC	GGC
AAG	ACG	CGC	GGC	GTG	GCG	ACG	CTG	TGC
ATC	GGC	GGG	GGC	GAA	GGC	ACC	GCA	GTG
GCA	CTC	GAT	TGC	TAT	AAG	AAC	CAT	GGC
TGG	GGA	CGC	CCG	ACA	ACA	GGC	GTC	CAC
CAG	CTT	TTT	TCA	TTC	CGA	CAA	CCC	GAA
CGA	ACA	ATG	CGT	AGA	GCA	GGA	GAT	TCC

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Table 4 (contd)

	Met	Arg	Pro	Ser	Ile	His	Arg	Thr
	ATG	CGC	CCA	TCC	ATC	CAC	CGC	ACA
Ala	Ile	Ala	Ala	Val	Leu	Ala	Thr	Ala
GCC	ATC	GCC	GCC	GTG	CTG	GCC	ACC	GCC
Phe	Val	Ala	Gly	Thr	Ala	Leu	Ala	Gln
TTC	GTG	GCG	GGC	ACC	GCC	CTG	GCC	CAG
Lys	Arg	Asp	Asn	Val	Leu	Phe	Gln	Ala
AAG	CGC	GAC	AAC	GTG	CTG	TTC	CAG	GCA
Ala	Thr	Asp	Glu	Gln	Pro	Ala	Val	Ile
GCT	ACC	GAC	GAG	CAG	CCG	GCC	GTG	ATC

NB. This fragment carries the leader sequence coding for the signal polypeptide, a part of the CPG_2 structural gene coding for the first 22 amino acids of the protein, the ATG start codon, the CPG_2 ribosome binding site (AGGA) and other components of the CPG_2 promoter region.

TABLE 5

Localisation of Signal Peptide - B-galactosidase Fusion Protein

	CPG_2/B-GAL	% LOCALISATION[a] AP	GAPDH	NADHOX
Periplasmic	50.3	97.3	3.4	0.4
Cytoplasmic	30.9	2.5	95.3	8.2
Membrane-bound	18.8	0.2	1.3	89.4

a = average results from 4 experiments

CPG_2/B-GAL = Carboxypeptidase G_2-B-galactosidase fusion protein

AP = Alkaline phosphatase

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase

NADHOX = NADH.O_2 Oxidoreductase

Example 7

Preparation of a recombinant plasmid, containing both the present leader sequence polynucleotide and the CPG_2 structural gene, able to replicate in E.coli and S.cerevisiae

A 2.03 kilobase BamHI fragment coding for the present signal polypeptide and the entire CPG_2 molecule was cloned in both orientations into the BamHI site of an E. coli/S. cerevisiae shuttle vector pROG5 (R.F. Sherwood and R.K. Gibson, The Molecular Biology of Yeast, 1979, Cold Spring Harbor Publications) to give plasmids pLEC3 and pLEC4 (Figure 7). These plasmids were transformed into S. cerevisiae strain LL20 by the lithium acetate induced transformation method described by Ito et al., J. Bact., 1983, 153, 163. Yields equivalent to 10-20 units/litre of culture volume were obtained regardless of gene orientation within the plasmid. Specific activity of the enzyme in total cell extracts was 0.2-0.3u/mg protein representing 0.005% soluble protein. This level of expression from the pseudomonad promotor in a yeast background is similar to the level found when the gene was reading from its own promotor in E.coli (0.01% soluble protein).

Localisation experiments have been performed on yeast cells carrying the above plasmids by sphaeroplasting the cells using standard techniques described by J.B.D. Beggs, Nature, 1978, 275, 105. Periplasmic enzymes, localised outside of the cell membrane, were released when the cell wall was removed. The osmotic stabiliser (1.2M sorbitol) was then replaced by 0.1M Tris-HCl buffer, pH 7.3 containing 0.2mM $ZnCl_2$ to lyse the sphaeroplasts and the whole centrifuged at 100,000 x g for 1 hour to separate proteins in the soluble cytoplasmic fraction from membrane bound proteins. The results in Table 6 demonstrate the presence of 64% of the CPG_2 activity in the periplasmic fraction and a further 16% associated with the cell membrane fraction.

TABLE 6

Localisation of CPG_2 in S. cerevisiae

	% CPG_2 activity
Periplasmic	64
Cytoplasmic	20
Membrane bound	16

CLAIMS

1. A recombinant DNA transfer vector comprising a leader sequence polynucleotide characterised in that the leader sequence polynucleotide codes for a signal polypeptide of formula I,

Met - Arg - Pro - Ser - Ile - His - Arg - Thr -
Ala - Ile - Ala - Ala - Val - Leu - Ala - Thr - I
Ala - Phe - Val - Ala - Gly - Thr

2. A recombinant DNA transfer vector according to claim 1 characterised in that the leader sequence polynucleotide is of formula II,

5'- ATG CGC CCA TCC ATC CAC CGC ACA
GCC ATC GCC GCC GTG CTG GCC ACC II
GCC TTC GTG GCG GGC ACC - 3'

3. A recombinant DNA transfer vector according to either claim 1 or claim 2 characterised in that the leader sequence polynucleotide is downstream of and in reading phase with a bacterial or a yeast promoter and a prokaryotic ribosome binding site.

4. A recombinant DNA transfer vector according to any one of claims 1 to 3 characterised in that the leader sequence polynucleotide is upstream of and in reading phase with a structural gene.

5. A recombinant DNA transfer vector according to claim 4 characterised in that the structural gene codes for human growth hormone, human insulin or human chorionic somatomammotropin.

6. A recombinant DNA transfer vector according to claim 4 characterised in that the structural gene codes for E.coli β - galactosidase.

7. A recombinant DNA transfer vector according to claim 4 characterised in that the structural gene codes for Pseudomonas carboxypeptidase G_2 (CPG_2).

8. A recombinant DNA transfer vector according to claim 7 comprising a polynucleotide of formula

	1								
	Met	Arg	Pro	Ser	Ile	His	Arg	Thr
5' -	ATG	CGC	CCA	TCC	ATC	CAC	CGC	ACA
		10							
	Ala	Ile	Ala	Ala	Val	Leu	Ala	Thr	Ala
	GCC	ATC	GCC	GCC	GTG	CTG	GCC	ACC	GCC
			20						
	Phe	Val	Ala	Gly	Thr	Ala	Leu	Ala	Gln
	TTC	GTG	GCG	GGC	ACC	GCC	CTG	GCC	CAG
				30					
	Lys	Arg	Asp	Asn	Val	Leu	Phe	Gln	Ala
	AAG	CGC	GAC	AAC	GTG	CTG	TTC	CAG	GCA
					40				
	Ala	Thr	Asp	Glu	Gln	Pro	Ala	Val	Ile
	GCT	ACC	GAC	GAG	CAG	CCG	GCC	GTG	ATC
						50			
	Lys	Thr	Leu	Glu	Lys	Leu	Val	Asn	Ile
	AAG	ACG	CTG	GAG	AAG	CTG	GTC	AAC	ATC
							60		
	Glu	Thr	Gly	Thr	Gly	Asp	Ala	Glu	Gly
	GAG	ACC	GGC	ACC	GGT	GAC	GCC	GAG	GGC
								70	
	Ile	Ala	Ala	Ala	Gly	Asn	Phe	Leu	Glu
	ATC	GCC	GCT	GCG	GGC	AAC	TTC	CTC	GAG
									80
	Ala	Glu	Leu	Lys	Asn	Leu	Gly	Phe	Thr
	GCC	GAG	CTC	AAG	AAC	CTC	GGC	TTC	ACG
	Val	Thr	Arg	Ser	Lys	Ser	Ala	Gly	Leu
	GTC	ACG	CGA	AGC	AAG	TCG	GCC	GGC	CTG
	90								
	Val	Val	Gly	Asp	Asn	Ile	Val	Gly	Lys
	GTG	GTG	GGC	GAC	AAC	ATC	GTG	GGC	AAG

	100							
Ile	Lys	Gly	Arg	Gly	Gly	Lys	Asn	Leu
ATC	AAG	GGC	CGC	GGC	GGC	AAG	AAC	CTG
		110						
Leu	Leu	Met	Ser	His	Met	Asp	Thr	Val
CTG	CTG	ATG	TCG	CAC	ATG	GAC	ACC	GTC
			120					
Tyr	Leu	Lys	Gly	Ile	Leu	Ala	Lys	Ala
TAC	CTC	AAG	GGC	ATT	CTC	GCG	AAG	GCC
				130				
Pro	Phe	Arg	Val	Glu	Gly	Asp	Lys	Ala
CCG	TTC	CGC	GTC	GAA	GGC	GAC	AAG	GCC
					140			
Tyr	Gly	Pro	Gly	Ile	Ala	Asp	Asp	Lys
TAC	GGC	CCG	GGC	ATC	GCC	GAC	GAC	AAG
						150		
Gly	Gly	Asn	Ala	Val	Ile	Leu	His	Thr
GGC	GGC	AAC	GCG	GTC	ATC	CTG	CAC	ACG
							160	
Leu	Lys	Leu	Leu	Lys	Glu	Tyr	Gly	Val
CTC	AAG	CTG	CTG	AAG	GAA	TAC	GGC	GTG
								170
Arg	Asp	Tyr	Gly	Thr	Ile	Thr	Val	Leu
CGC	GAC	TAC	GGC	ACC	ATC	ACC	GTG	CTG
Phe	Asn	Thr	Asp	Glu	Glu	Lys	Gly	Ser
TTC	AAC	ACC	GAC	GAG	GAA	AAG	GGT	TCC
180								
Phe	Gly	Ser	Arg	Asp	Leu	Ile	Gln	Glu
TTC	GGC	TCG	CGC	GAC	CTG	ATC	CAG	GAA
	190							
Glu	Ala	Lys	Leu	Ala	Asp	Tyr	Val	Leu
GAA	GCC	AAG	CTG	GCC	GAC	TAC	GTG	CTC
		200						
Ser	Phe	Glu	Pro	Thr	Ser	Ala	Gly	Asp
TCC	TTC	GAG	CCC	ACC	AGC	GCA	GGC	GAC

			210					
Glu	Lys	Leu	Ser	Leu	Gly	Thr	Ser	Gly
GAA	AAA	CTC	TCG	CTG	GGC	ACC	TCG	GGC
				220				
Ile	Ala	Tyr	Val	Gln	Val	Asn	Ile	Thr
ATC	GCC	TAC	GTG	CAG	GTC	AAC	ATC	ACC
					230			
Gly	Lys	Ala	Ser	His	Ala	Gly	Ala	Ala
GGC	AAG	GCC	TCG	CAT	GCC	GGC	GCC	GCG
						240		
Pro	Glu	Leu	Gly	Val	Asn	Ala	Leu	Val
CCC	GAG	CTG	GGC	GTG	AAC	GCG	CTG	GTC
							250	
Glu	Ala	Ser	Asp	Leu	Val	Leu	Arg	Thr
GAG	GCT	TCC	GAC	CTC	GTG	CTG	CGC	ACG
								260
Met	Asn	Ile	Asp	Asp	Lys	Ala	Lys	Asn
ATG	AAC	ATC	GAC	GAC	AAG	GCG	AAG	AAC
Leu	Arg	Phe	Asn	Trp	Thr	Ile	Ala	Lys
CTG	CGC	TTC	AAC	TGG	ACC	ATC	GCC	AAG
270								
Ala	Gly	Asn	Val	Ser	Asn	Ile	Ile	Pro
GCC	GGC	AAC	GTC	TCG	AAC	ATC	ATC	CCC
	280							
Ala	Ser	Ala	Thr	Leu	Asn	Ala	Asp	Val
GCC	AGC	GCC	ACG	CTG	AAC	GCC	GAC	GTG
		290						
Arg	Tyr	Ala	Arg	Asn	Glu	Asp	Phe	Asp
CGC	TAC	GCG	CGC	AAC	GAG	GAC	TTC	GAC
			300					
Ala	Ala	Met	Lys	Thr	Leu	Glu	Glu	Arg
GCC	GCC	ATG	AAG	ACG	CTG	GAA	GAG	CGC
				310				
Ala	Gln	Gln	Lys	Lys	Leu	Pro	Glu	Ala
GCG	CAG	CAG	AAG	AAG	CTG	CCC	GAG	GCC

					320			
Asp	Val	Lys	Val	Ile	Val	Thr	Arg	Gly
GAC	GTG	AAG	GTG	ATC	GTC	ACG	CGC	GGC
						330		
Arg	Pro	Ala	Phe	Asn	Ala	Gly	Glu	Gly
CGC	CCG	GCC	TTC	AAT	GCC	GGC	GAA	GGC
							340	
Gly	Lys	Lys	Leu	Val	Asp	Lys	Ala	Val
GGC	AAG	AAG	CTG	GTC	GAC	AAG	GCG	GTG
								350
Ala	Tyr	Tyr	Lys	Glu	Ala	Gly	Gly	Thr
GCC	TAC	TAC	AAG	GAA	GCC	GGC	GGC	ACG
Leu	Gly	Val	Glu	Glu	Arg	Thr	Gly	Gly
CTG	GGC	GTG	GAA	GAG	CGC	ACC	GGC	GGC
360								
Gly	Thr	Asp	Ala	Ala	Tyr	Ala	Ala	Leu
GGC	ACC	GAC	GCG	GCC	TAC	GCC	GCG	CTC
	370							
Ser	Gly	Lys	Pro	Val	Ile	Glu	Ser	Leu
TCA	GGC	AAG	CCA	GTG	ATC	GAG	AGC	CTG
		380						
Gly	Leu	Pro	Gly	Phe	Gly	Tyr	His	Ser
GGC	CTG	CCG	GGC	TTC	GGC	TAC	CAC	AGC
			390					
Asp	Lys	Ala	Glu	Tyr	Val	Asp	Ile	Ser
GAC	AAG	GCC	GAG	TAC	GTG	GAC	ATC	AGC
				400				
Ala	Ile	Pro	Arg	Arg	Leu	Tyr	Met	Ala
GCG	ATT	CCG	CGC	CGC	CTG	TAC	ATG	GCT
					410			
Ala	Arg	Leu	Ile	Met	Asp	Leu	Gly	Ala
CGC	CGC	CTG	ATC	ATG	GAT	CTG	GGC	GCC
Gly	Lys							
GGC	AAG	TGA - 3'						

9. A recombinant DNA transfer vector according to any preceding claim characterised in that the transfer vector is a plasmid.

10. A recombinant DNA transfer vector according to claim 9 whenever taken together with claim 7 having the designation pNM1, pNM111, pNM14, pNM21, pNM22, pNM31, pNM32 or pIEC3.

11. A microorganism transformed by a transfer vector characterised in that the transfer vector is a recombinant DNA transfer vector according to claim 1.

12. A microorganism according to claim 11 characterised in that the transfer vector is a recombinant DNA transfer vector according to claim 4.

13. A microorganism according to either claim 11 or claim 12 which is a bacterium of the species E.coli, Pseudomonas or Bacillus or a yeast of the species Saccharomyces cerevisiae.

14. A process for the preparation of a gene product characterised by

(a) culturing a microorganism according to claim 12 in a culture medium to produce the gene product in the culture medium or the periplasmic space of the microorganism, and

(b) isolating the gene product from the culture medium or the periplasmic space of the microorganism.

15. A process according to claim 14 characterised in that the gene product is Pseudomonas carboxypeptidase G_2 or E.coli β - galactosidase.

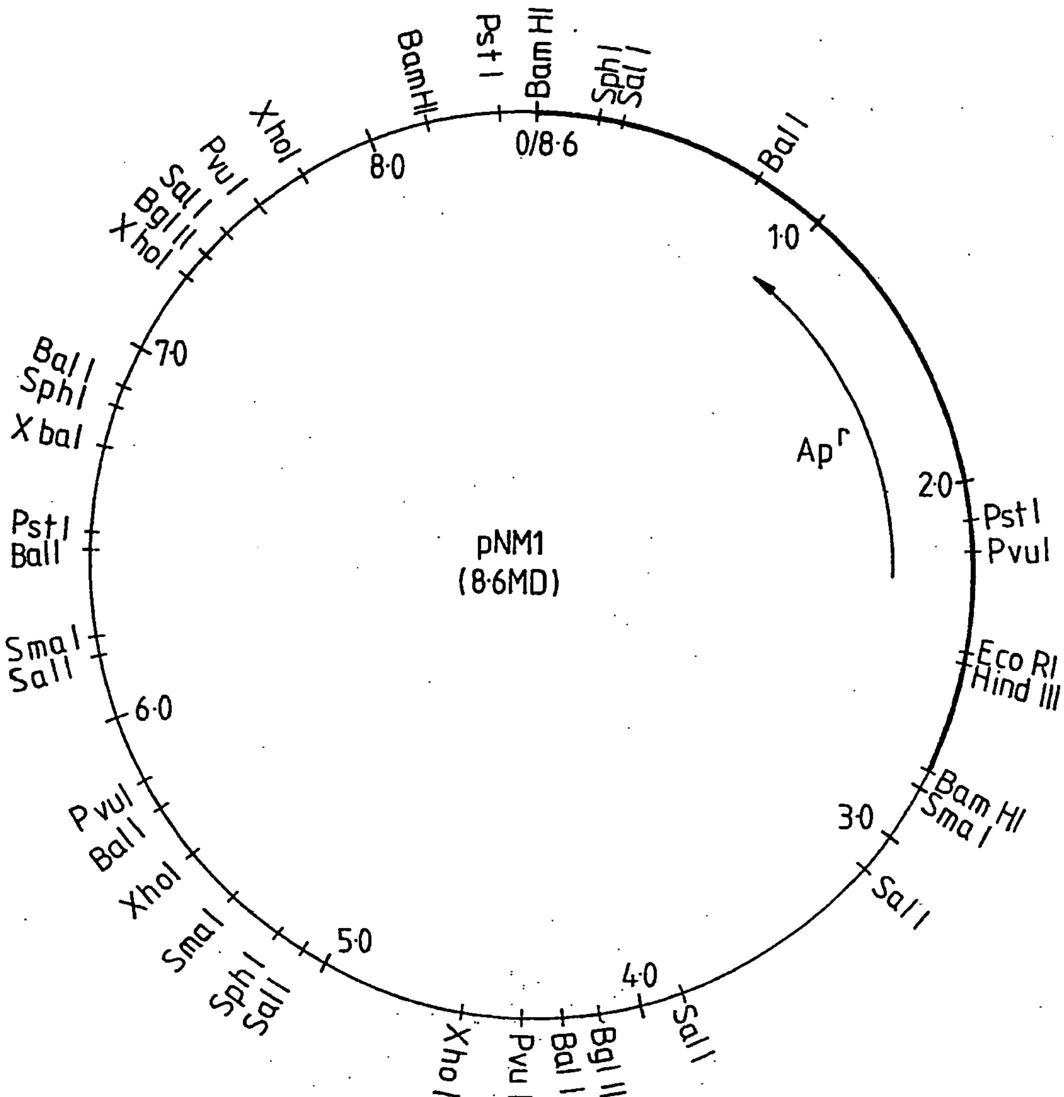
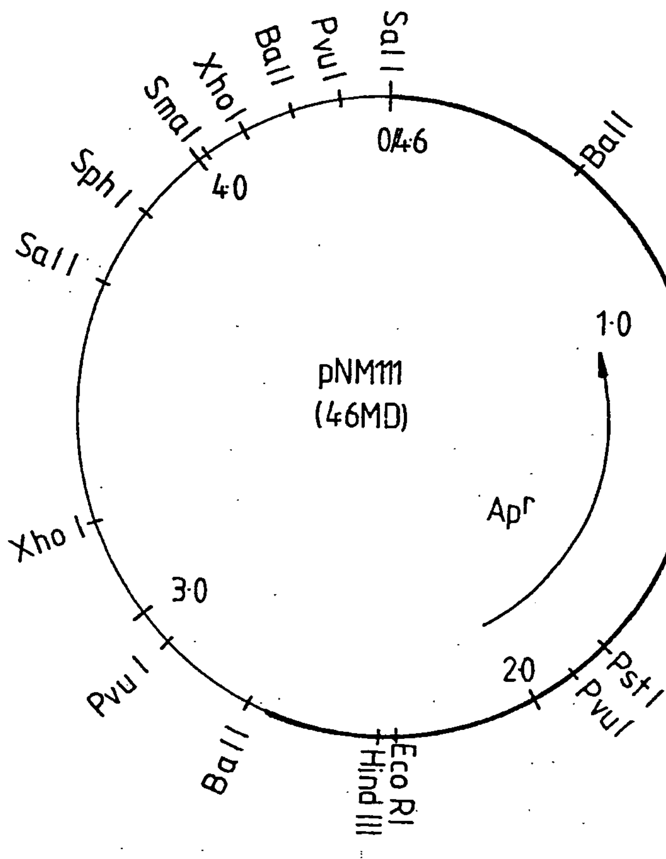


Fig.1.

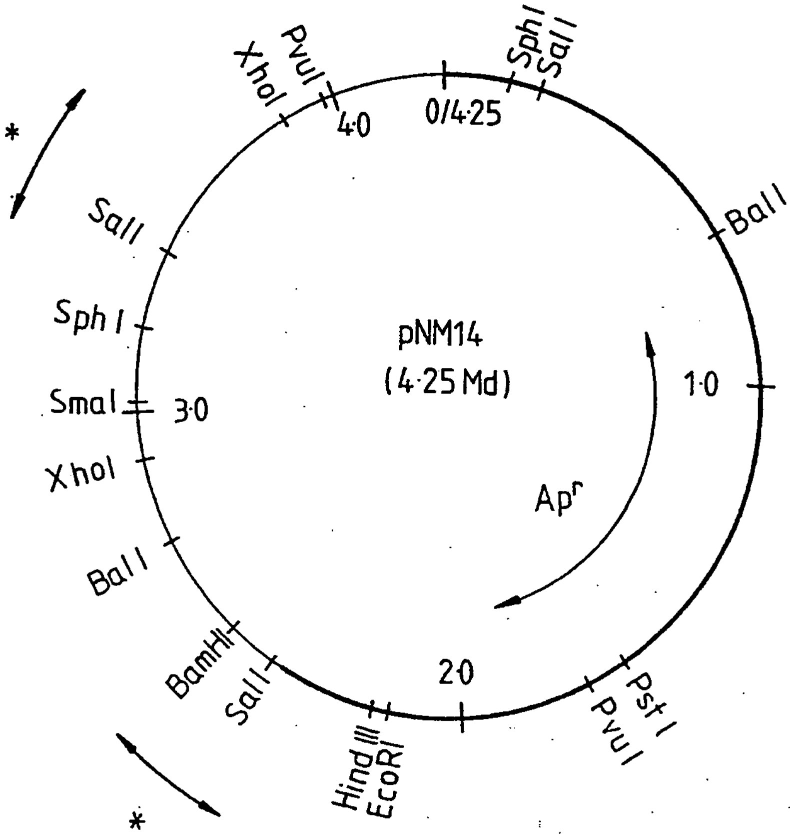
RESTRICTION ENZYME MAP OF pNM1

——— REPRESENTS pBR322



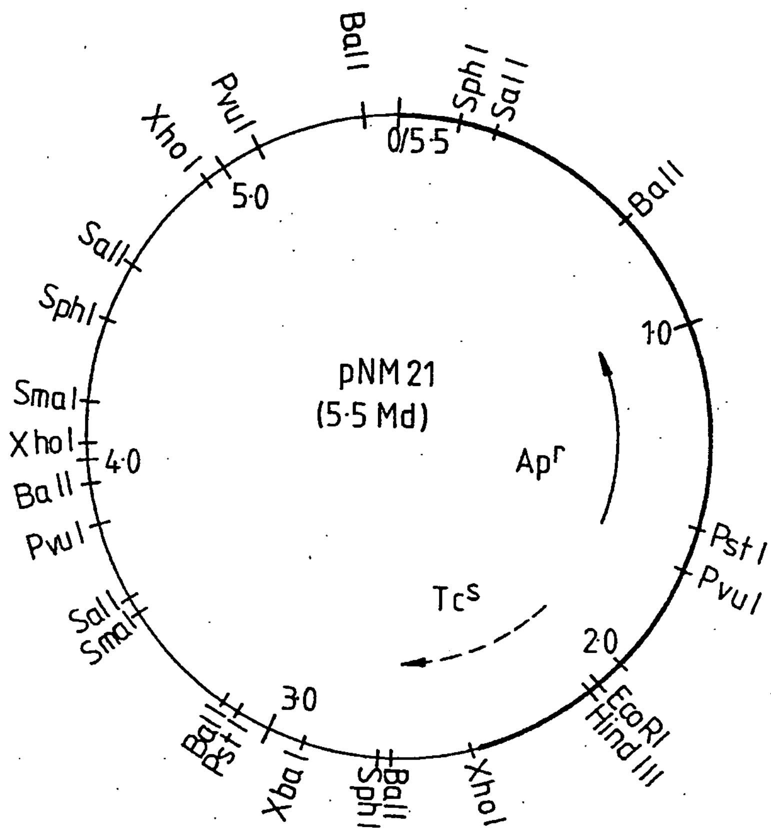
RESTRICTION ENZYME MAP OF pNM111

Fig.2.



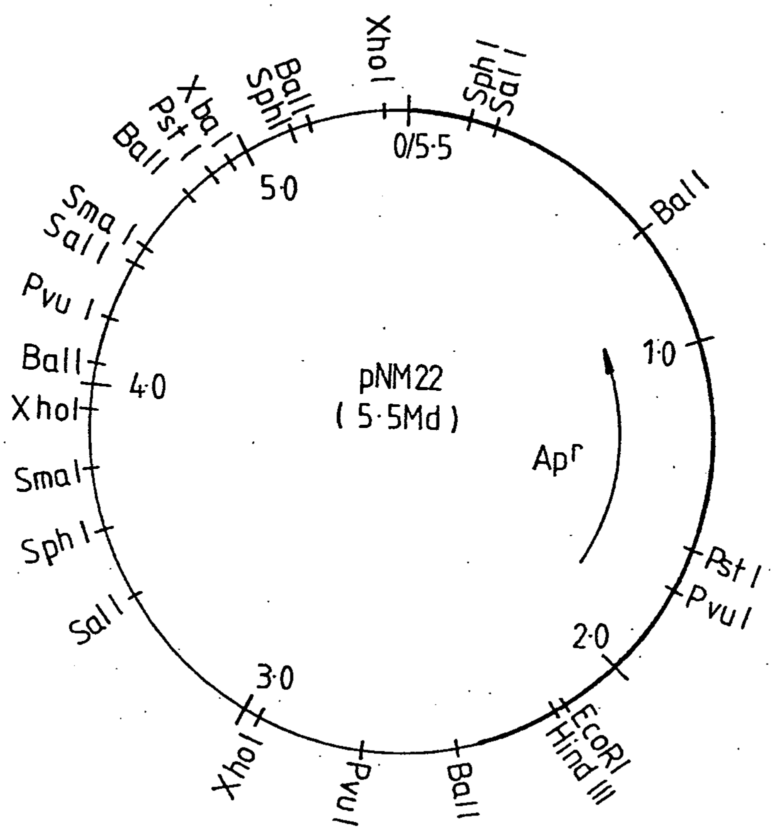
RESTRICTION ENZYME MAP OF pNM14

Fig.3.



RESTRICTION ENZYME MAP OF pNM21

Fig.4.



RESTRICTION ENZYME MAP OF pNM22

Fig.5.

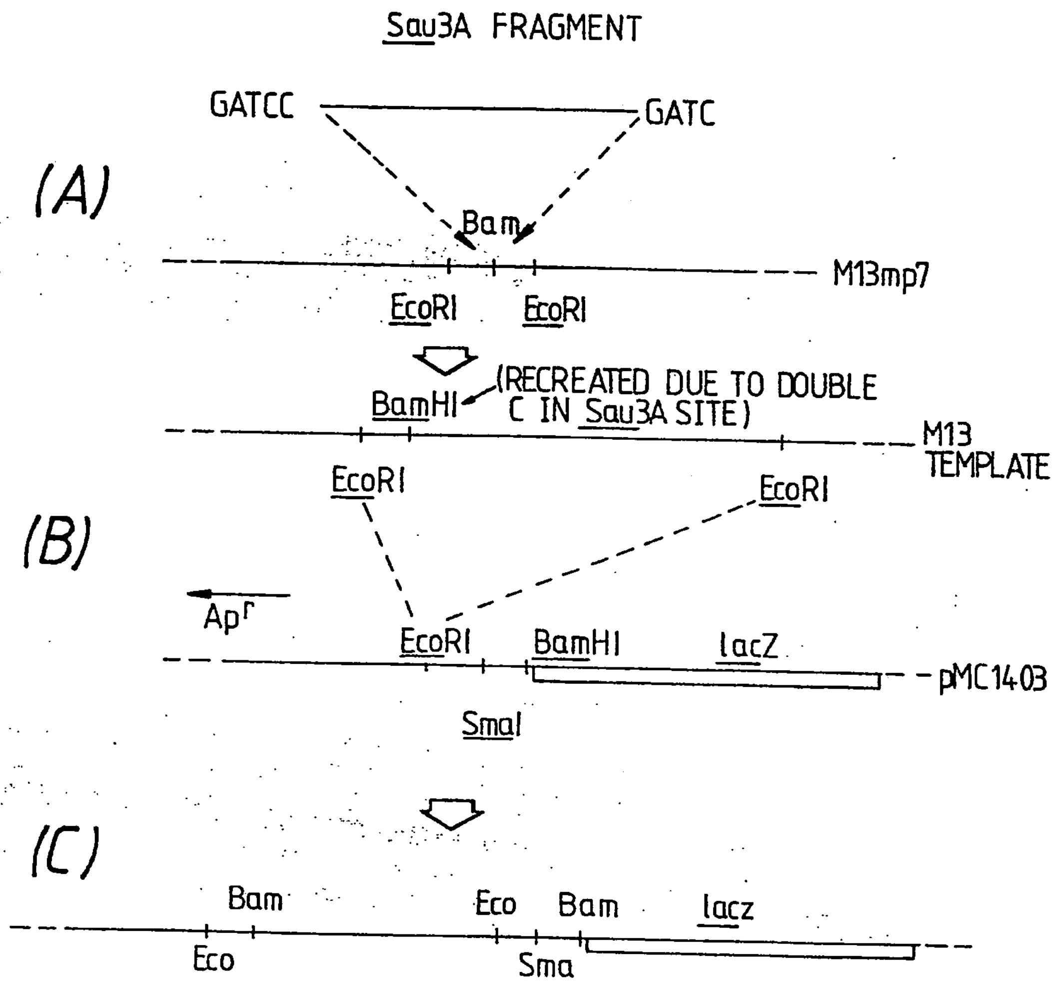
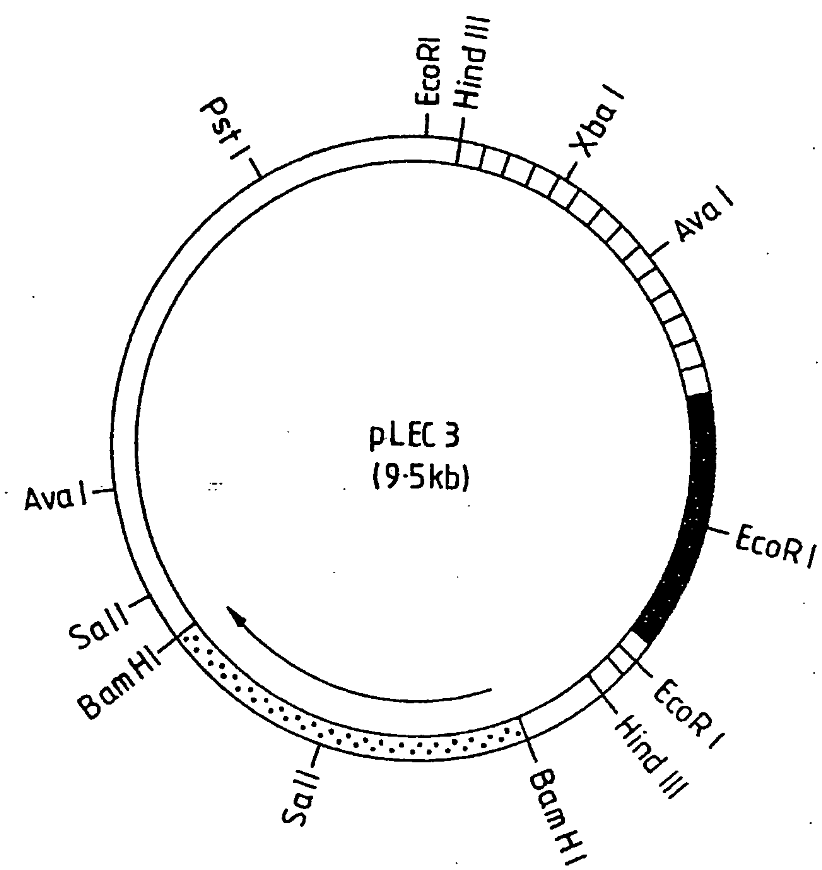

Sau3A FRAGMENT
GATCC
GATC
(A)
Bam
M13mp7
EcoRI
EcoRI
BamHI
(RECREATED DUE TO DOUBLE C IN Sau3A SITE)
M13 TEMPLATE
EcoRI
EcoRI
(B)
Apr
EcoRI
BamHI
lacZ
pMC1403
SmaI
(C)
Bam
Eco
Bam
lacz
Eco
Sma

Fig.6.



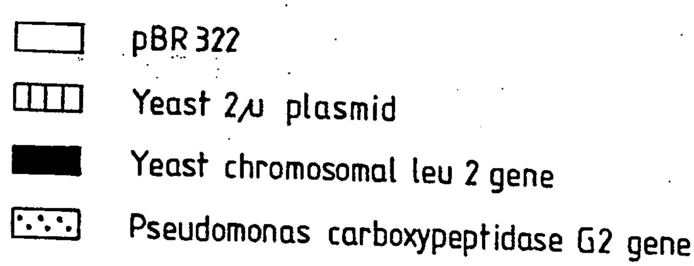


Fig.7.

RESTRICTION ENZYME MAP OF pLEC 3

European Patent Office

EUROPEAN SEARCH REPORT

0121352

Application number

EP 84301468.9

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. ³)
A,D	EP - A2 - 0 001 931 (GENENTECH, INC.) * Claims 1,6 * --	1,5	C 12 N 15/00 C 12 P 19/34 C 07 C 103/52 C 12 P 21/00 C 12 N 9/48 C 12 N 9/38// C 12 R 1/19 C 12 R 1/38 C 12 R 1/07 C 12 R 1/865
A	EP - A2 - 0 049 619 (ELI LILLY AND COMPANY) * Claims 1,3 *	1,5	
D,A	& GB-A-2 007 675 ----		
			TECHNICAL FIELDS SEARCHED (Int. Cl. ³)
			C 12 N C 12 P C 07 C 103/00

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	02-08-1984	WOLF

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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D : document cited in the application
L : document cited for other reasons
& : member of the same patent family, corresponding document

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